Viral load of human papilloma virus 16 as a determinant for development of cervical carcinoma in situ: a nested case-control study

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Summary

Background Infection with certain types of human papillomavirus (HPV), which is common among young women, increases the risk of cervical cancer. However, less than 1% of young women positive for oncogenic types of HPV develop cervical cancer. We investigated whether the amount of HPV DNA is a useful predictor of progression to cervical carcinoma in situ.

Methods We estimated the amount of HPV 16 DNA by a PCR that uses the 5′-exonuclease (Taqman) method, in 478 women with cervical carcinoma in situ and 608 individually matched controls. To adjust for differences in the amount of genomic DNA between samples, we estimated the amount of a nuclear gene (β-actin). We studied multiple smears (total 3835 archived samples) from each woman, taken over periods of up to 26 years, that covered normal cytology to development of cervical cancer.

Findings The risk of cervical carcinoma in situ increased with the amount of HPV 16 DNA. Analysis of the first smear from each woman, collected a mean of 7·8 years before cancer diagnosis, showed that women with the 20% highest amount of HPV 16 DNA were at a 60-fold higher risk of developing cervical carcinoma in situ than women negative for HPV 16. The first smear samples were classified as normal by squamous-cell cytology.

Interpretation Analysis of the amount of HPV DNA can predict cancer risk at a stage when current screening methods are uninformative. Testing for the amount of HPV 16 DNA during gynaecological health checks might strikingly improve our ability to distinguish between infections that have a high or low risk of progressing into cervical cancer.

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Introduction

Infection by certain types of human papillomavirus (HPV), especially HPV 16 and HPV 18, increases the risk of cervical cancer. Although HPV infection is common among young women, less than 1% of those positive for oncogeneic types of HPV develop cervical cancer.[1,2] Therefore, the presence of HPV has a low predictive value. Several studies have suggested that the amount of HPV could be an important factor for progression from HPV infection to cervical cancer.[3–10] Without the availability of a method to estimate the amount of HPV in clinical samples, however, no study has been able to address the importance of viral load for cancer risk.

We did a nested case-control study of the relation between amount of HPV DNA and development of carcinoma in situ by analysis of archived cervical-smear samples.

Methods

Patients

We studied samples collected during routine gynaecological health checks from women with cervical cancer and individually matched controls from the general population.[11] We selected women from a cohort who lived in Uppsala County, Sweden, between 1969 and 1995.[11] To identify all eligible cases of cervical carcinoma in situ, we merged information from the organised screening programme, recorded from 1969 to 1995, with data from the National Cancer Registry. We included women who met the following entry criteria: born in Sweden, younger than 50 years at entry, and first smear classified as normal by squamous-cell cytology. For each case, we randomly selected from the study cohort five separate controls, matched with each other by date of entry to the cohort (within 90 days). Controls had to be still alive without having developed cervical or invasive cancer before the date of diagnosis for their matched case. Cases and controls were matched for age and time (calendar date) at which the first smear was collected. The first smear for controls also had to be classified as normal by cytology on squamous cells. The study was approved by the local ethics committees.

DNA extraction and assay

HPV DNA was purified from archived Papanicolaou-stained smears according to established procedures.[12,13] The procedure consisted of xylene incubation, destaining in 99·5% ethanol, treatment with proteinase K (60°C, minimum 1 h), and subsequent protein precipitation by saturated ammonium acetate. The DNA in the supernatant was recovered with ethanol, the pellet washed with 70% ethanol, dried, and dissolved in 200 μL TE-low (10 mmol/L Tris hydrochloric acid, pH 7·4, 0·1 mmol/L edetic acid).

We estimated the amount of HPV DNA by quantitative PCR that used the 5′-exonuclease assay and real-time detection of the accumulation of fluorescence (Taqman).[14–16] The Taqman assay uses a non-extendible DNA probe with a fluorescent dye attached to the 5′-end and a second fluorescent dye linked to the 3′-end. When the probe is intact, the emission of the 5′ dye is quenched by the 3′ dye. During PCR, the 5′ to 3′ exonuclease activity of the DNA polymerase is used to cleave the

hybridisation probe during the extension phase, resulting in a release of fluorescence proportional to the amount of amplicon generated. This method can be used to estimate the number of DNA molecules present in a sample before PCR. The estimate is achieved by comparison of the threshold cycle number (C_t) for the sample (which represents the PCR cycle number at which the fluorescent signal exceeds the baseline signal) with the C_t for a range of samples with known starting copy numbers. This method has been used to estimate the amount of several genetic targets, including oncogenic types of HPV.[15] The Taqman HPV assay is based on DNA amplification of a 180 bp fragment of the E1 open reading frame in the presence of an HPV-16-specific hybridisation probe, and allows for quantification of the amount of HPV 16 DNA in a sample.[16] To adjust for variation in the number of cells in different samples, we did quantification of a fragment of the β-actin nuclear gene, also by the Taqman method.

We did the PCR amplification in a 25 μL volume, and included 50 mmol/L potassium chloride, 10 mmol/L Tris hydrochloric acid (pH 8·4), 10 mmol/L edetic acid, 60 nmol/L of 6-carboxy-X-rhodamine (Rox) used as passive reference dye, 5 mmol/L magnesium chloride, 0·2 mmol/L each of dATP, dCTP, and dGTP, 0·4 mmol/L dUTP, 124 ng/μL bovine serum albumin, 0·5 U uracile N′ glycosylase, 1·25 U DNA polymerase PE Biosystems, Foster City, CA, USA), 0·25 μmol/L HPV E1 5′ primer, 0·5 μmol/L HPV E1 3′ primer, and 1–5% of the DNA obtained from a cervical smear. Our 5′ primer mix for the E1 ORF consisted of two primers (HPVE116L 5′-TAC AGG TTC TAA AAC GAA AGT-3′ and 5′-HPVE118L TGC ATG TTT TAA AAC GAA AGT-3′) and the 3′ primer mix of three primers (HPVE116R 5′-TTC CAC TTC AGT ATT GCC ATA-3′, HPVE118R 5′-TTC CAC TTC AGA ACA GCC ATA-3′ and HPVE1RE 5′-TRY RKG MNY TAA AAC GAA AGT-3′).[15] A probe specific to HPV 16 (FAM-5′-ATA ATC TCC TTT TTG CAG CTC TAC TTT GTT TTTp-3′-TAMRA; FAM=6-carboxyfluorescein, TAMRA=6-carboxy-tetramethylrhodamine) was used at a concentration of 0·1 pmol per amplification reaction, and the fluorescence was detected by an ABI Prism 7700, Sequence Detection System (Perkin-Elmer Inc). The amplification ramp included two hold programmes— 2 min at 50°C and 10 min at 95°C—followed by a two-step PCR cycle with a melting step for 15 s at 95°C and an annealing for 1 min at 55°C, for a total of 50 cycles. The threshold cycle was calculated by the Sequence Detection System (SDS) software (version 1.6.3) and the baseline automatically set to 10 SD above background in the first 3–15 cycles.

For the detection of β-actin, we used a commercially available kit (Perkin-Elmer, kit N8080230) that uses as reverse primer (5′-TCA CCC ACA CTG TGC CCA TCT ACG A-3′ and a forward primer (5′-CAG CGG AAC CGC TCA TTG CCA ATG G-3′. The probe (5′-[FAM] ATG CCC- X[TAMRA]- CCC CCA TGC CAT CCT GCG Tp-3′) is directed towards nucleotide positions 2171–2196. The amplification ramp included two hold programmes—2 min at 50°C and 10 min at 95°C—followed by a two-step PCR cycle with a melting step for 15 s at 95°C and an annealing for 1 min at 60°C, for a total of 50 cycles.

Statistical analyses

We estimated odds ratios, with 95% CI and p values, by conditional logistic regression (PHREG procedure in SAS software). The positive predictive value for development of cervical carcinoma in situ in relation to the amount of HPV 16 DNA in the first β-actin-positive smear taken from each woman was estimated from the result of an unconditional logistic-regression analysis, adjusted for the variables in the matching criteria, the time to diagnosis, and the amount of genomic DNA (β-actin). We adjusted for the matching criteria by inclusion of the interaction between age at first smear (<20 years, 20–24 years, 25–29 years, and ≥30 years) and calendar period of first smear (before 1970, 1970–1974, 1975–1979, and 1980 or after). Times to diagnosis were grouped (0–2 years, 2–5 years, 5–10 years, and ≥10 years), as were amounts of genomic DNA (34·78≥C_t β-actin>34·78), by division of the distribution at the point when the effect modification was the strongest. To estimate absolute risk, we added an offset variable to the model for each combination of age and calendar period of first smear. This offset variable indicated the inverse probability of being selected for the study, giving control status. This probability was estimated simply as the number of controls in the case-control study divided by the number of available controls for each combination of the matching criteria. The number of available controls fulfilling the entry criteria was obtained from a database comprising all women with smears taken in the County of Uppsala.

	Cases (n=478)	Controls (n=608)
β-actin-positive smears		
Total	2081	1754
Median (range)	4 (1–17)	2 (1–14)
75th percentile	6	4
Distribution of β-actin C_t		
Minimum	19·96	24·61
25th percentile	35·04	35·44
Median	37·30	37·58
75th percentile	39·10	39·24
Maximum	49·08	49·30
HPV-16-positive smears		
total	871	117
No positive smear	190	509
≥1 positive smear	288	99
Distribution of HPV 16 C_t		
Minimum	23·13	31·49
25th percentile	34·74	40·08
Median	37·59	43·88
75th percentile	40·92	46·77
Maximum	49·72	49·90

Table 1: **Baseline characteristics**

Results

The nested case-control study included 504 cases and 662 controls. For some controls, only one smear was available during the follow-up period. Therefore, a second matched control for each of those was randomly chosen from the original pool of controls to increase the statistical power. 158 second controls were included. To confirm the cancer diagnosis of cases, the histological samples (complete cone or small biopsy) were reassessed by an experienced pathologist. After cytological and histological review, the study included 499 cases, 499 first controls, and 158 second controls. Subsequently, controls that had undergone hysterectomy before the diagnosis of their corresponding case were excluded. Remaining were 495 cases, 495 first controls, and 154 second controls.

462 smears from cases and 386 smears from controls were negative for β-actin, presumably because of too little DNA in the sample. An additional 17 cases and controls were, therefore, excluded. The remaining 478 complete matched sets of 478 cases (2081 smears) and 608 controls (478 first controls and 130 second controls [1754 smears]) had at least one β-actin-positive smear for each woman in the set and were used in the analyses (table 1).

871 (42%) smears from cases and 117 (7%) from controls were positive for HPV 16 (table 1). The median HPV 16 C_t values were lower for cases than controls (37·59 *vs* 43·88, table 1, figure 1). Because of the way in which the C_t values are calculated, there is an inverse relation between C_t values and HPV DNA amount (copy number). The lower median C_t value for the smears from cases, therefore, correspond to a higher HPV DNA copy number.

The C_t values for HPV 16 did not change over time, and the difference between cases and controls was

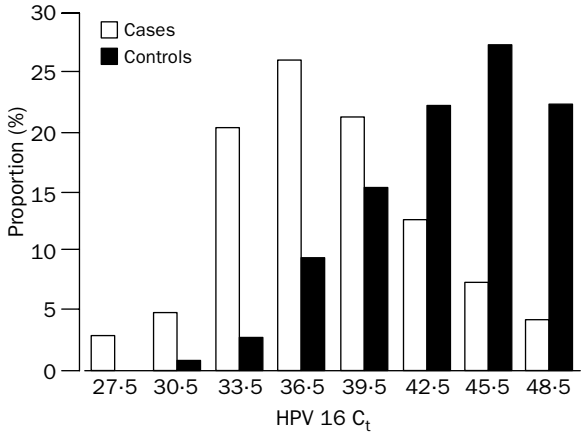

Figure 1: **HPV 16 C_t values for case and control smears**
HPV 16 C_t values were separated into eight groups; mid-group values are shown.

constant for all calendar periods (figure 2). The higher amount of HPV 16 DNA in cases than in controls cannot, therefore, be explained by variation in DNA quality over calendar time. The C_t values for β-actin did not differ significantly for cases or controls over time (figure 2) and, therefore, changes in the procedures for preparation of the smear samples and reagents used for preparation of PAP smears did not affect the findings. Also, since the median β-actin C_t value for cases (37·30) was similar to that for the controls (37·58), DNA quality did not differ systematically between cases and controls (table 1).

HPV DNA amount and cancer risk

Many of the cases' smears were taken as part of diagnostic work-up. The likelihood of detecting an HPV infection may be related to the number of smears taken. To achieve comparability between cases and controls, we excluded smears taken later than 1 year before diagnosis from the analysis of the odds ratio. The mean number of remaining smears per woman was similar for cases and controls (2·9 *vs* 2·2). The odds ratio, based on the mean HPV 16 C_t of all smear samples from a woman, was significant for each quintile of the distribution (defined from the joint distribution of HPV-16-positive smears of cases and controls) and increased with increasing amounts of HPV DNA (lower HPV 16 C_t, table 2). After adjustment for differences in amount of genomic DNA between smears by use of the β-actin C_t values, the odds ratio of developing cervical carcinoma in situ for women with the highest amount of viral DNA (HPV 16 C_t <36·66, odds ratio 68·8 [95% CI 5·8–299·6]) was almost 70-fold higher than for women negative for HPV 16 (table 2).

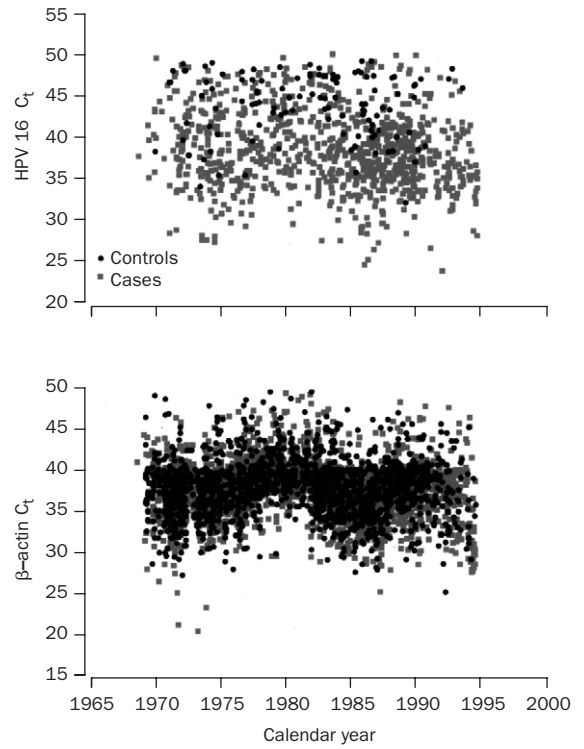

Figure 2: **HPV 16 C_t values and distribution of β-actin C_t values for case and control smears by calendar year of preparation of smears**

Categories*	Mean HPV C_t	
	Cases/controls	Odds ratio (95% CI)†
HPV negative	212/464	1·0
HPV positive, C_t 44·8–50·0	27/32	2·0 (1·1–3·8)
HPV positive, C_t 41·25–44·8	41/18	4·4 (2·3–8·3)
HPV positive, C_t 38·99–41·25	48/12	8·1 (3·8–17·3)
HPV positive, C_t 36·66–38·99	52/7	18·7 (7·1–49·5)
HPV positive, C_t <36·66	58/2	68·8 (15·8–299·6)

*Calculated on each 20th percentile of distribution of mean HPV 16 C_t value for each woman. †Adjusted for β-actin.

Table 2: **Odds ratios (95% CI) of developing cervical carcinoma in situ in relation to mean estimates of HPV 16 viral load per β-actin-positive woman**

The odds ratios for the first β-actin positive smear, taken on average 7·8 years before diagnosis and classified normal by cytology, also increased with increasing amount of HPV DNA, and were significant for all quintiles except the first (table 3). Since only one smear per woman was included in this analysis, the relation between amount of viral DNA and risk of cancer avoided any bias because of differences in number of smears between cases and controls. Also, the findings for the first smear from each woman cannot result from asymmetric sampling of cases and controls, since the controls were individually matched to their case for time of first smear. The consistency of the increase in odds ratio with amount of HPV DNA between the estimates, based on the mean and first smear, supports the validity of the observed relation.

Given the significant odds ratio for high amounts of HPV 16 DNA, we calculated positive predictive values for different amounts of HPV 16 DNA to predict risk of developing cervical cancer. The positive predictive value estimates the absolute risk of developing cervical carcinoma in situ for the entire study according to the amount of HPV 16 DNA in the first β-actin-positive

Categories*	HPV C_t of first smear	
	Cases/controls	Odds ratio (95% CI)†
HPV negative	354/578	1·0
HPV positive, C_t 45·26–50·0	16/15	1·9 (0·8–4·2)
HPV positive, C_t 42·08–45·26	23/7	7·2 (2·7–19·1)
HPV positive, C_t 38·7–42·08	28/3	22·8 (5·5–95·0)
HPV positive, C_t 35·9–38·7	27/4	18·9 (5·5–64·9)
HPV positive, C_t <35·9	30/1	59·0 (7·5–462·2)

*Categories are calculated on each 20th percentile of distribution of C_t HPV 16 value for for first smear for each woman. †Adjusted for β-actin.

Table 3: **Odds ratios of developing cervical carcinoma in situ in relation to HPV 16 viral load in first smear among β-actin-positive women**

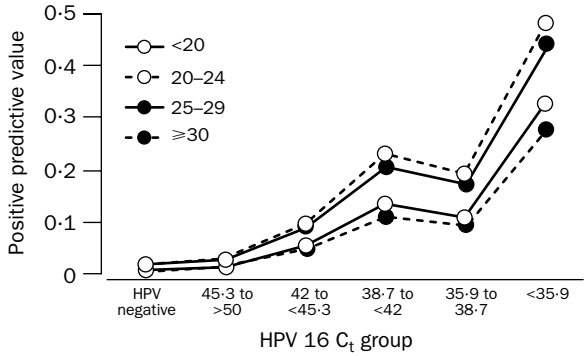

Figure 3: **Positive predictive value at different quintiles of distribution of HPV 16 C_t values**
Results presented are for women with a first smear diagnosed in 1970–74, but similar results were obtained for the other calendar periods. Categories of HPV 16 C_t values are as in table 3. Analyses are for smears with high amounts of nuclear DNA (⩾34·78 β-actin C_t).

smear only. Smears were stratified into two groups with high and low β-actin C_t values. The results presented are for the calendar period 1970–74 and for women followed up for 5–10 years, but similar results were obtained for the other calendar periods. The positive predictive value increased with the amount of HPV 16 DNA in all age-groups, and for the two subgroups of high and low amounts of β-actin (figure 3). For example, for women aged 20–24 years who had high amounts of genomic DNA (β-actin C_t ⩾34·78) and with HPV 16 C_t values lower than 35·9, the positive predictive value was more than 48%. This value should be compared with the probability of 1·7% of a woman in the same age-group having a negative result.

Discussion

High amounts of HPV 16 DNA is a major risk factor for development of cervical carcinoma in situ. The amount of viral DNA could vary between individuals because of environmental factors,[11,17–20] genetic factors, or both. Several environmental factors, such as smoking, use of oral contraceptives, and sequence variation in oncogenic HPV types have been purported to affect the risk of infection. The amount of viral DNA may also reflect inherent differences between individuals in response to HPV 16 infection. Genetic susceptibility to cervical tumours has been shown.[21] Putative genetic cofactors include genes affecting the immune response to infection or viral DNA replication.[22–25] The amount of HPV DNA seems to predict the risk of developing cervical carcinoma before any cytological alterations are visible, and certainly long before the appearance of tumours.[27] This finding is consistent with the existence of inherent susceptibility factors that exert effects at or close to the time of infection. The importance of the immune reaction to HPV infection is supported by the association of persistent infection with an increased risk of cervical cancer.[26] Although beyond the resolution of the present method, study of the intracellular distribution of HPV genomes would be interesting, especially whether the distribution of HPV genomes differs between samples with high and low titres of HPV. If HPV is more localised to a smaller number of cells in the high-titre samples, analysis of the intracellular distribution might be useful as a cancer predictor.

Measurements of the amount of HPV 16 DNA might be of use in estimating the probability of an infection progressing to cervical carcinoma in situ. The estimated positive predictive value of our findings was too low for the test to be directly applicable as a single test for predicting cancer risk, except for in women with the highest amount of HPV DNA. Further developments of the test may, however, increase the positive predictive value. First, we studied a single oncogenic type of HPV, albeit the most common. Given that there is a positive relation between viral load and cancer risk for other oncogenic HPV types, use of an assay covering 95–99% of the HPV types associated with cancer, rather than only HPV 16, which is present in 40–60% of the tumours, would substantially increase the positive predictive value. Second, we have analysed archival smear samples, which, because of poor DNA quality, are likely to yield a distribution that is skewed towards higher HPV C_t values (lower HPV amounts). Analysis of fresh samples may yield lower HPV C_t values, which would allow better differentiation between samples with high and low HPV titres and, potentially, a higher positive predictive value. Given a high positive predictive value for HPV load, a quantitative test for HPV DNA has the potential to predict cancer risk at a stage when the presently applied screening method, based on microscope examination of the cytology sample, is uninformative. The analysis of HPV DNA amount seems to have a higher specificity than the typing for presence or absence of HPV in smear samples.

Contributors

Agnetha Josefsson developed the quantitative PCR-based HPV analysis, did the HPV titre determinations, wrote the main part of the paper, and participated in the statistical analysis and interpretation of the data. Patrik Magnusson analysed the data, participated in the interpretation of the results, and wrote the statistical parts of the paper. Nathalie Ylitalo participated in the design of the study and the organisation of cervical-smear collection. Pernilla Qwarforth-Tubbin extracted the DNA from cervical smears, determined the HPV titre together with Agnetha Josefsson, and participated in the data analysis. Per Kragh Andersson and Mads Melbye contributed to the statistical analysis of the data and the interpretation of the results. Hans-Olov Adami, the principal investigator, designed the study and coordinated the research. Ulf B Gyllensten, the coprincipal investigator, conceived the idea to study HPV titres, had the overall responsibility for the development of the system for determining HPV titre, and for the statistical analysis and preparation of the paper.

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